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# Effects of zinc oxide on the attachment of *Staphylococcus aureus* strains

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**Abstract**

We examined the attachment of *Staphylococcus aureus* to plastic tissue-culture coverslips after incubation for 24 h. The attachment to coverslips was weaker in rabbit plasma with 5% zinc oxide (ZnO) than in the control rabbit plasma without ZnO ($P < 0.01$). Plasma coagulation by *S. aureus* strains was not detected in plasma with 5% ZnO after incubation for 24 h. The membranous structure (an immature biofilm) was formed on the coverslips by *S. aureus* cells in plasma after incubation for 24 h. The colony counts of *S. aureus* cells on the membranous structures were lower in plasma with 5% ZnO, plasma with 0.2% hinokitiol, plasma with 5% ZnO + 0.2% hinokitiol, plasma with cefdinir at 4 minimum inhibitory concentration (MIC) and plasma with levofloxacin at 4 MIC, than in the control plasma after incubation for 24 h ($P < 0.01$). The colonies on the membranous structures completely disappeared in the case of plasma with 5% ZnO and 0.2% hinokitiol. The colony counts on membranous structures were lower in plasma with cefdinir at 4 MIC or levofloxacin at 4 MIC containing 5% ZnO than in plasma with cefdinir at 4 MIC or levofloxacin at 4 MIC only, ($P < 0.05$). The MICs of hinokitiol against *S. aureus* strains peaked at an MIC distribution of 16–32 μg/ml. The peak shifted to below 1 μg/ml by adding 5% ZnO in agar plate method. The results suggest that the attachment of *S. aureus* cells to the coverslips is suppressed in the presence of 5% ZnO and that antistaphylococcal activities of cefdinir, levofloxacin and hinokitiol increase in the presence of 5% ZnO. 

*Keywords:* *Staphylococcus aureus*; Atopic dermatitis; Attachment; Zinc oxide

## 1. Introduction

Eczematous skin lesions of atopic dermatitis (AD) are usually densely colonized with *Staphylo-*

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*coccus aureus* [1–5] and a correlation between the severity of eczematous lesions and the density of *S. aureus* colonization has been demonstrated [3,5]. We reported that hinokitiol (HK) ointment (white petrolatum with 0.2% HK and 5% zinc oxide (ZnO)) was useful for the reduction of *S. aureus* in AD patients [6]. *Staphylococcus aureus* cells isolated from impetigo, furuncle and AD lesions produced a biofilm in plasma on plastic tissue-culture coverslips after incubation for 72 h at 37°C [7,8]. The purpose of our present study is to examine the in vitro effects of ZnO on the attachment of *S. aureus* cells to the plastic tissue-culture coverslips with or without HK, cefdinir (CFDN), or levofloxacin (LVFX) and the coagulation of plasma by *S. aureus* cells.

## 2. Materials and methods

### 2.1. Bacterial strains

*Staphylococcus aureus* strain (our laboratory's No. 96–78), isolated from eczematous AD lesion, was used to examine plasma coagulation and attachment by *S. aureus*. No. 96–78 strain belonging to coagulase type VII (latex agglutination method). The minimum inhibitory concentration (MIC) of oxacillin against strain No. 96–78 was 1 $\mu$g/ml.

Three other *S. aureus* strains (one, coagulase type VII; two, coagulase type III) isolated from eczematous AD lesions and five *S. aureus* strains isolated from skin infections (staphylococcal impetigo and furuncle) were also used to examine plasma coagulation. Two *S. aureus* strains isolated from impetigo lesions were used to examine attachment. Four *S. aureus* strains isolated from impetigo belonged to coagulase type V and produced exfoliative toxin A [9]. One *S. aureus* strain isolated from a furuncle lesion belonged to coagulase type IV.

No. 96–78 strain and fifteen other *S. aureus* strains (seven, coagulase type VII; eight, coagulase type III), isolated from AD lesions, were used in the experiment on the MIC of hinokitiol (HK) ($C_{10}H_{10}O_2$) (Wako, Osaka, Japan).

### 2.2. Bacterial suspension for inoculation

*Staphylococcus aureus* strains were grown in 8 ml of tryptic soy broth (TSB; Nissui Pharmaceutical, Tokyo, Japan) at 37°C for 24 h without shaking. Following incubation, the bacterial cells were harvested by centrifugation at 9000 × *g* for 10 min at 4°C. The bacterial cells were resuspended in sterile saline solution and centrifuged as described above; the process was repeated three times. The washed bacteria were resuspended in polypropylene microtubes (1 ml: Iuchi BioSystems, Tokyo, Japan) and tissue-culture dishes (35 × 10 mm style) (Becton Dickinson, New Jersey) ($n = 3$).

### 2.3. In vitro attachment of *S. aureus* cells to the plastic tissue-culture coverslips

We examined the attachment of *S. aureus* cells to Celldesk® plastic tissue-culture coverslips (Sumitomo Bakelite, Tokyo, Japan). The coverslips were incubated with *S. aureus* cells in culture medium for 24 h at 37°C and were then gently washed with sterile saline solution (1 ml) ten times. The coverslips were suspended in 4 ml of sterile saline solution and sonicated (Ultrasonics, Model W-225R) at 60% power for 30 s at 4°C. The stripped organisms were counted as colony forming units (CFU) using the 10-fold dilution method.

### 2.4. *S. aureus* cell attachment to coverslips with or without ZnO

Cell suspension of strain No. 96–78, which contained $2.2 \times 10^8$ CFU, was used. Cells were inoculated to 2.5 ml each of rabbit plasma (Denka Seiken, Tokyo, Japan), rabbit plasma with the addition of 5% zinc oxide (ZnO) (Wako) and rabbit plasma with the addition of 1% ZnO, with 1.77 $cm^2$ of coverslips in each tissue-culture dish ($n = 3$). After incubation for 24 h at 37°C, the number of *S. aureus* cells attached to each coverslip was determined and checked for plasma coagulation. After incubation for 24 h at 37°C, when compared with the growth rate of cells in TSB without ZnO, the growth rate of No. 96–78 strain

cells in TSB with 5% ZnO decreased to 1/6, and the growth rate of *S. aureus* cells in TSB with 1% ZnO decreased to 1/2.

Cell suspensions of the two *S. aureus* strains isolated from impetigo lesions which contained 4.4 or $6.7 \times 10^8$ CFU were used. Cells were inoculated to 2.5 ml each of rabbit plasma and rabbit plasma with 5% ZnO, with 1.77 $cm^2$ of coverslips in each tissue-culture dish ($n = 3$). After incubation for 24 h at 37°C, the number of *S. aureus* cells attached to each coverslip was determined.

### *2.5. S. aureus cell count on membranous structure under various conditions*

Cell suspension of strain No. 96–78, which contained 2.5 or $5.9 \times 10^8$ CFU, was used to inoculate to 2.5 ml, rabbit plasma with 1.77 $cm^2$ of coverslips in tissue-culture dishes. After incubation for 24 h at 37°C, membranous structures were formed on the coverslips. Half of the membranous structures ($n = 3$) were used in each inoculation to 2.5 ml of rabbit plasma, rabbit plasma with 0.56% methanol (Katayama Chemical, Osaka, Japan), rabbit plasma with 5% ZnO, rabbit plasma with 0.2% HK, and rabbit plasma with 5% ZnO + 0.2% HK. The other half of the membranous structures ($n = 3$) were used in each inoculation to 2.5 ml of rabbit plasma, rabbit plasma with 5% ZnO, rabbit plasma with cefdinir (CFDN) (Fujisawa Pharmaceutical, Osaka, Japan) at 4 MIC with or without 5% ZnO and rabbit plasma with levofloxacin (LVFX) (Daiichi Pharmaceutical, Tokyo, Japan) at 4 MIC with or without 5% ZnO. After incubation for 24 h at 37°C, the number of *S. aureus* cells attached to each coverslip was determined and checked for plasma coagulation. CFDN and LVFX were used as the antimicrobial agents, as they have strong antistaphylococcal activity with many antibiotics and are commonly used in the dermatological field. The MIC of HK, in Mueller-Hinton (MH) (Difco, Detroit) agar plate against strain No. 96–78, was 16 $\mu$g/ml. The MIC of HK in MH agar plate with 5% ZnO against strain No. 96–78 was $\geq 1$ $\mu$g/ml. Hinokitiol was dissolved with a small aliquot as methanol, so plasma with 0.2% HK contained 0.56% methanol. The MIC of CFDN or LVFX against strain No. 96–78 was 0.5 $\mu$g/ml.

### *2.6. Observation of plasma coagulation under various conditions*

Cell suspensions of the nine *S. aureus* strains which contained $2.0–8.8 \times 10^7$ CFU were used. Cells were inoculated to 1 ml each of rabbit plasma, rabbit plasma with 5% ZnO, rabbit plasma with 1% ZnO, rabbit plasma with 0.1% ZnO and rabbit plasma with 5% zinc chloride ($ZnCl_2$) (Ishizu Seiyaku, Osaka, Japan) in microtubes. Plasma coagulation was observed after incubation for 6, 18 and 24 h at 37°C.

### *2.7. Observation of MIC of hinokitiol under various conditions*

The MICs of HK against sixteen *S. aureus* strains were examined in MH agar, MH agar with 5% ZnO and MH agar with 5% $ZnCl_2$ using the agar plate method (inoculum size; $10^6$ CFU/ml).

### *2.8. Statistical methods*

Data analysis was conducted using a *t*-test for unpaired comparisons.

## 3. Results

### *3.1. S. aureus attachment to coverslip with or without ZnO*

Table 1 shows the colony counts of *S. aureus* strain No. 96–78 attached to coverslips and detection of plasma coagulation in rabbit plasma, rabbit plasma with 5% ZnO and rabbit plasma with 1% ZnO after incubation for 24 h at 37°C. The attachment to coverslips was weaker in plasma with 5% ZnO and 1% ZnO than in control plasma without ZnO ($P < 0.01$).

Two *S. aureus* strains isolated from impetigo lesions were inoculated to rabbit plasma and rabbit plasma with 5% ZnO. The colony counts attached to coverslips after incubation for 24 h at 37°C were noted. When compared with the num-

bers of cells in plasma without ZnO, the numbers of *S. aureus* cells in plasma with 5% ZnO decreased to about 1/1000 ($P<0.01$).

### 3.2. *S. aureus* cell count on membranous structure under various conditions

Table 2 shows the colony counts of *S. aureus* strain No. 96–78 on membranous structure and detection of plasma coagulation in rabbit plasma, rabbit plasma with 0.56% methanol, rabbit plasma with 5% ZnO, rabbit plasma with 0.2% HK and rabbit plasma with 5% ZnO + 0.2% HK after incubation for 24 h at 37°C. The colony counts on membranous structures were lower in plasma with 5% ZnO, plasma with 0.2% HK and plasma with 5% ZnO + 0.2% HK, than in the control plasma without any of them ($P<0.01$). The colonies on the membranous structures disappeared completely in the case of plasma with 5% ZnO and 0.2% HK.

Table 3 shows the colony counts of *S. aureus* strain No. 96–78 on membranous structure and detection of plasma coagulation in rabbit plasma, rabbit plasma with 5% ZnO, rabbit plasma with CFDN at 4 MIC with or without 5% ZnO and rabbit plasma with LVFX at 4 MIC with or without 5% ZnO, after incubation for 24 h at 37°C. The colony counts on membranous structures were lower in plasma with 5% ZnO, plasma with CFDN at 4 MIC or LVFX at 4 MIC, than in the control plasma without any of them ($P<0.01$). The colony counts on membranous structures were lower in plasma with CFDN at 4 MIC or LVFX at 4 MIC containing 5% ZnO than in plasma with CFDN at 4 MIC or LVFX at 4 MIC only ($P<0.05$).

### 3.3. Observation of plasma coagulation under various conditions

Table 4 shows the detection of plasma coagulation by nine *S. aureus* strains in rabbit plasma,

Table 1
Colony counts of *S. aureus* strain No. 96–78[a]

| Medium | Colony counts (CFU) (mean ± S.D.) | Plasma coagulation |
|---|---|---|
| Plasma | $1.7\times10^{7}\pm0.45\times10^{7}$ | + |
| Plasma with 5% ZnO | $1.5\times10^{4}\pm3.7\times10^{3}$* | − |
| Plasma with 1% ZnO | $1.3\times10^{4}\pm1.8\times10^{3}$* | − |

Cell suspension of strain No. 96–78 which contained $2.2\times10^{8}$ CFU was used. Cells were inoculated to 2.5 ml each of rabbit plasma, rabbit plasma with 5% ZnO and rabbit plasma with 1% ZnO, with 1.77 cm of the coverslips in tissue-culture dishes. After incubation for 24 h at 37°C, the number of *S. aureus* cells attached to each coverslip was determined and checked if plasma coagulated.
+, Positive; −, negative.
$n=3$.
[a] Attached to coverslips and detection of plasma coagulation in plasma, plasma with 5% ZnO and plasma with 1% ZnO after incubation for 24 h.
* Significantly different from colony count in plasma ($P<0.01$).

Table 2
Colony counts of *S. aureus* strain No. 96–78[a]

| Medium | Colony counts (CFU) (mean ± S.D.) | Plasma coagulation |
|---|---|---|
| Plasma | $1.1\times10^{7}\pm0.25\times10^{7}$ | + |
| Plasma with 0.56% methanol | $5.0\times10^{6}\pm0.71\times10^{6}$ | + |
| Plasma with 5% ZnO | $5.8\times10^{5}\pm1.6\times10^{5}$* | − |
| Plasma with 0.2% hinokitiol | $8.9\times10^{4}\pm0.81\times10^{4}$* | + |
| Plasma with 5% ZnO + 0.2% hinokitiol | ND* | − |

Cell suspension of strain No. 96–78 which contained $5.9\times10^{8}$ CFU was used in inoculation to 2.5 ml of rabbit plasma with 1.77 cm$^2$ of coverslips in tissue-culture dishes ($n=3$). After incubation for 24 h at 37°C, membranous structures were formed on the coverslips. The membranous structures were used in each inoculation to 2.5 ml of rabbit plasma, rabbit plasma with 0.56% methanol, rabbit plasma with 5% ZnO, rabbit plasma with 0.2% hinokitiol and rabbit plasma with 5% ZnO + 0.2% hinokitiol. After incubation for 24 h at 37°C, the number of *S. aureus* cells attached to each coverslip was determined and checked if plasma coagulated.
+, Positive; −, negative; ND, not detectable.
[a] On membranous structure and detection of plasma coagulation in plasma, plasma with 0.56% methanol, plasma with 5% ZnO, plasma with 0.2% hinokitiol and plasma with 5% ZnO + 0.2% hinokitiol after incubation for 24 h.
* Significantly different from colony count in plasma ($P<0.01$).

rabbit plasma with 5% ZnO, rabbit plasma with 1% ZnO, rabbit plasma with 0.1% ZnO and rabbit plasma with 5% $ZnCl_2$, after incubation for 6, 18, and 24 h at 37°C. Plasma coagulation by four *S. aureus* strains isolated from AD lesions was not detected in inoculated plasma with 1% ZnO or 5% $ZnCl_2$ after incubation for 6, 18, and 24 h. Plasma coagulation by five *S. aureus* strains isolated from skin infections (impetigo and furuncle) was not detected in inoculated plasma with 5% ZnO or 5% $ZnCl_2$ after incubation for 6, 18, and 24 h. In contrast, for four of the five strains, plasma coagulation was detected in inoculated plasma with 1% ZnO after incubation for 24 h.

Table 3
Colony count of *S. aureus* strain No. 96–78 on membranous structure and detection of plasma coagulation[a]

| Medium | Colony counts (CFU) (mean ± S.D.) | Plasma coagulation |
|---|---|---|
| Plasma | $4.9 \times 10^6 \pm 0.78 \times 10^6$ | + |
| Plasma with 5% ZnO | $1.8 \times 10^5 \pm 0.4 \times 10^5$* | – |
| Plasma with CFDN at 4 MIC | | |
| With 5% ZnO | $4.8 \times 10^4 \pm 0.85 \times 10^4$ | – |
| Without 5% ZnO | $5.1 \times 10^5 \pm 1.7 \times 10^5$ | + |
| Plasma with LVFX at 4 MIC | | |
| With 5% ZnO | $4.5 \times 0^4 \pm 0.33 \times 10^4$ | – |
| Without 5% ZnO | $6.2 \times 10^5 \pm 2.0 \times 10^5$ | + |

Cell suspension of strain No. 96–78, which contained $2.5 \times 10^8$ CFU was used in inoculation to 2.5 ml rabbit plasma with 1.77 $cm^2$ of coverslips in tissue-culture dishes ($n = 3$). After incubation for 24 h at 37°C, membranous structures were formed on the coverslips. The membranous structures were used in each inoculation to 2.5 ml of rabbit plasma, rabbit plasma with 5% ZnO, rabbit plasma with CFDN at 4 MIC with or without 5% ZnO and rabbit plasma with LVFX at 4 MIC with or without 5% ZnO. After incubation for 24 h at 37°C, the number of *S. aureus* cells attached to each coverslip was determined and checked if plasma coagulated.

+, Positive; –, negative.

[a] In plasma, plasma with 5% ZnO, plasma with cefdinir (CFDN) at 4 MIC with or without 5% ZnO, and plasma with levofloxacin (LVFX) at 4 MIC with or without 5% ZnO after incubation for 24 h.

* Significantly different from colony count in plasma ($P < 0.01$).

** $P < 0.05$.

### 3.4. MIC of hinokitiol under various conditions

The MICs for HK against sixteen *S. aureus* strains showed a peak MIC distribution at 16–32 μg/ml, but the peak shifted to 1 μg/ml ≥ by adding 5% ZnO or $ZnCl_2$.

## 4. Discussion

Bacterial sphingomyelinases from *S. aureus* and *Bacillus cereus* are stimulated in the presence of $Mg^{2+}$ and inhibited by $Ca^{2+}$ [10–14]. Ikezawa et al. reported that sphingomyelinase of *B. cereus* was strongly and almost irreversibly inhibited by $Zn^{2+}$ [15]. Baine reported that phospholipase C from *Legionella pneumophilia* was inhibited by $Zn^{2+}$ and $Cu^{2+}$ [16]. Some phospholipase C produced by gram-positive bacteria have been shown to be zinc-metalloenzymes [17,18], which are activated by divalent metal ions such as $Zn^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ [19]. Tawara et al. reported that the hemolytic activity of phospholipase C from *Pseudomonas aeruginosa* was completely inhibited in the presence of 1 m mol/l $Zn^{2+}$ and inactivation of the enzymes by $Zn^{2+}$ was not reversed by concomitant addition of other divalent metal ions [19]. *Staphylococcus* is rather resistant to high concentration of NaCl in culture medium [20]. We reported that the attachment of *S. aureus* cells isolated from AD lesion to the coverslips was suppressed in the presence of 10% NaCl (pH 5.6) or 10% sea salts (containing 0.372% $Mg^{2+}$ ($P < 0.01$) [21]. Plasma coagulation by four *S. aureus* strains isolated from AD lesions was not detected in plasma with 10% NaCl or 10% sea salts after incubation for 60 h at 37°C [21]. We reported that the attachment of two *S. aureus* strains isolated from impetigo lesions to the coverslips was suppressed in the presence of 10% NaCl or 10% sea salts ($P < 0.05$) [21]. We also

Table 4
Detection of plasma coagulation by nine *S. aureus* strains[a]

| Medium | Hours after inoculation | | |
|---|---|---|---|
| | 6 | 18 | 24 |
| 4]Four *S. aureus* strains isolated from atopic dermatitis lesions | | | |
| Plasma | 3/4 | 4/4 | 4/4 |
| Plasma with 5% ZnO | 0/4 | 0/4 | 0/4 |
| Plasma with 1% ZnO | 0/4 | 0/4 | 0/4 |
| Plasma with 0.1% ZnO | 1/4 | 3/4 | 3/4 |
| Plasma with 5% $ZnCl_2$ | 0/4 | 0/4 | 0/4 |
| Five *S. aureus* strains isolated from infections (impetigo and furuncle) | | | |
| Plasma | 5/5 | 5/5 | 5/5 |
| Plasma with 5% ZnO | 0/5 | 0/5 | 0/5 |
| Plasma with 1% ZnO | 0/5 | 3/5 | 4/5 |
| Plasma with 0.1% ZnO | 3/5 | 5/5 | 5/5 |
| Plasma with 5% $ZnCl_2$ | 0/5 | 0/5 | 0/5 |

Cell suspensions of the nine *S. aureus* strains which contained $2.0-8.8 \times 10^7$ CFU were used. Cells were inoculated to 1 ml each of rabbit plasma, rabbit plasma with 5% ZnO, rabbit plasma with 1% ZnO, rabbit plasma with 0.1% ZnO and rabbit plasma with 5% $ZnCl_2$ in microtubes. Plasma coagulation was observed after incubation for 6, 18, and 24 h at 37°C.

[a] In plasma, plasma with 5% ZnO, plasma with 1% ZnO, plasma with 0.1% ZnO and plasma with 5% $ZnCl_2$ after incubation for 6, 18 and 24 h.

examined the plasma coagulation by five *S. aureus* strains isolated from skin infections (impetigo and furuncle) by adding 10% NaCl or 10% sea salts, or not. For one of the five strains, plasma coagulation was detected in plasma with 10% NaCl after incubation for 12 h at 37°C and for four of the five strains, plasma coagulation was detected in plasma with 10% sea salts in the same condition [21]. In this experiment, the attachment of *S. aureus* cells, isolated from AD lesions and impetigo lesions, to the coverslips was suppressed in the presence of 5% ZnO. Plasma coagulation by these strains was not detected in plasma with 5% ZnO after incubation for 24 h at 37°C. Because plasma coagulation was not detected in plasma with 5% $ZnCl_2$ after incubation for 24 h at 37°C, $Zn^{2+}$ seems to have inhibited plasma coagulation.

The first step in the establishment of an infection is the attachment of bacteria to tissues [7]. Fibrinogen contributed to the attachment of *S. aureus* cells to coverslips in plasma [21]. The attachment of *S. aureus* cells to coverslips, conversion of fibrinogen to fibrin and the production of an abundant glycocalyx by *S. aureus* cells are the minimum requirement for the production of biofilm on the coverslip [7]. No. 96–78 strain cells produced a mature biofilm in plasma on the coverslip after 72 h at 37°C [8]. So the membranous structure, which was formed on coverslips by No. 96–78 strain cells in rabbit plasma after incubation for 24 h at 37°C, may be an immature biofilm. In this experiment, anti-staphylococcal activities of CFDN, LVFX and HK against membranous structures increased on addition of 5% ZnO in plasma. If plasma coagulation does not occur in the presence of 5% ZnO, the adherence of *S. aureus* cells may become weaker and staphylococcal infections may be suppressed.

The MICs for HK against 73 *S. aureus* strains showed a peak MIC distribution at 64 $\mu$g/ml, but the peak shifted to 2 $\mu$g/ml on addition of 200 $\mu$g/ml (0.02%) of ZnO or $ZnCl_2$ in microdilution method [6]. The MICs for HK against *S. aureus* strains isolated from AD lesions were also reduced on addition of 5% ZnO or $ZnCl_2$ in agar plate. We have already reported that the difference in coagulase types of *S. aureus* strains isolated from impetigo lesions and those from

AD lesions; coagulase types I and V were dominant in impetigo lesions, while types III and VII were dominant in AD lesions [22]. The different degrees of plasma coagulation in this study and the pervious study [21] similarly reflect the difference in coagulase types of the *S. aureus* strains isolated from infections (impetigo and furuncle) and those isolated from AD lesions. The growth rate of No. 96–78 strain cells in TSB with 5% ZnO decreased to, at most, 1/6, compared with the growth rate of cells in TSB without ZnO after incubation for 24 h at 37°C (Section 2.4). So our results indicate that the attachment of *S. aureus* cells to coverslips greatly decreased in the presence of 5% ZnO (Tables 1 and 2) and that antistaphylococcal activities of CFDN, LVFX, and HK increased in the presence of 5% ZnO (Tables 2 and 3). These phenomena may be useful to suppress *S. aureus* colonization or infection in patients with AD. We propose that HK ointment containing 5% ZnO and 0.2% HK is most effective in eliminating *S. aureus* cell attached to the skin lesions of AD patients.

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